

Rescue of polyglutamine-mediated cytotoxicity by double-stranded RNA-mediated RNA interference

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RNA interference (RNAi) is a mechanism that appears to control unwanted gene expression in a wide range of species. In *Drosophila*, RNAi is most effectively induced by double-stranded RNAs (dsRNAs) of over ~80 nucleotides (nt) and in mammalian cells an RNAi-like inhibition of gene expression has been shown to be mediated by dsRNAs of ~21–23 nt. To test if RNAi can be used to specifically down-regulate a human disease-related transcript we have used *Drosophila* and human tissue culture models of the dominant genetic disorder spinobulbar muscular atrophy (SBMA). A variety of different dsRNAs were assessed for the ability to inhibit expression of transcripts that included a truncated human androgen receptor (*ar*) gene containing different CAG repeat lengths (16–112 repeats). In *Drosophila* cells, dsRNAs corresponding to non-repetitive sequences mediated a high degree of sequence-specific inhibition, whereas RNA duplexes containing CAG repeat tracts only induced gene-specific inhibition when flanking *ar* sequences were included; dsRNAs containing various lengths of CAG repeats plus *ar* sequences were unable to induce allele-specific interference. In mammalian cells we tested sequence-specific small dsRNAs of 22 nt; these rescued the toxicity and caspase-3 activation induced by plasmids expressing a transcript encoding an expanded polyglutamine tract. This study demonstrates the feasibility of targeting a transcript associated with an important group of genetic diseases by RNAi.

INTRODUCTION

Gene silencing mediated by double-stranded RNAs (dsRNAs) has emerged as an important mechanism regulating exogenous and potentially endogenous gene expression. Termed post-transcriptional gene silencing in plants and fungi, and RNA interference (RNAi) in other organisms (1), this process is mediated by dsRNA and induces the sustained down-regulation of the target gene corresponding to the dsRNA (2,3) as a result of degradation of the target mRNA. Whereas most work to date has been performed in lower eukaryotes, there is also evidence for RNAi in mouse zygotes and embryos (4,5) and in both mouse and human cells in culture where dsRNAs of ~21–23 nucleotides (nt) have been shown to specifically inhibit gene expression (6,7). Because of the efficacy and ease with which RNAi can be applied, this mechanism has been rapidly exploited in *Caenorhabditis elegans* and *Drosophila* as a reverse genetics tool (8–10). The genetic treatment of a number of disease states including infectious disease, tumor development and dominant genetic disorders would be another application for a mechanism that specifically down-regulates gene expression. Previous methodologies that have been

exploited to achieve this goal have included antisense technology and catalytic ribozymes (11); however, the efficacy of these approaches has been mixed, particularly *in vivo*. Given the effectiveness of dsRNA-mediated gene silencing, we wanted to assess whether this mechanism could be used to specifically down-regulate a human disease-related transcript.

At least eight human neurodegenerative disorders, including Huntington's disease (HD) and spinobulbar muscular atrophy (SBMA) (Kennedy's disease) are caused by expansion of trinucleotide (CAG) repeats (12). SBMA is an X-linked, adult onset motor neuronopathy caused by a progressive loss of lower motor neurons in the spinal cord and brain stem and sensory neurons in the dorsal root ganglia. The genetic defect underlying SBMA is an expansion of a polymorphic trinucleotide CAG repeat in the first exon of the androgen receptor (*ar*) gene. The normal range of repeats is 11–35 glutamines; individuals with SBMA have an expanded CAG repeat length encoding an increased polyglutamine (polyQ) tract of 38–62 residues (13). The androgen receptor (AR) is a ligand-activated transcription factor and its biological functions are well characterized. However, the physiological functions of the polyQ

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tract in the N-terminal transactivation domain of the protein, as well as the mechanism by which this expansion leads to neurodegeneration, are unknown, though most evidence suggests that polyglutamine expansion in the AR protein results in a toxic gain-of-function.

Aggregation of mutant polyQ proteins is a feature present in all of the polyglutamine disorders (14). In individuals with SBMA, intranuclear aggregates that contain the AR protein are present in motor neurons of the brain stem and spinal cord as well as in other tissues that normally express this protein (15,16). Similar findings have been reported in transgenic mouse models of HD and SBMA and patients with HD (17–20). These protein aggregates are insoluble, and in cell culture are associated with abnormal proteolytic processing of the AR protein (21–23). Though protein mis-folding and aggregation is seen in most of the polyQ diseases there is still some debate as to whether aggregate formation is directly neurotoxic or a by-product of disease progression. Some studies have suggested that protein aggregation or nuclear inclusions are neither necessary nor sufficient to initiate disease (24–26); however, there is also evidence that peptides containing expanded polyQ tracts are intrinsically cytotoxic (27–31). There are several mechanisms by which these abnormal proteins could be acting pathogenically, including, sequestration of critical cellular factors into protein aggregates, inhibition of key cellular pathways such as ubiquitination, and the induction of proteolysis. Evidence for the last of these possible mechanisms is increasing with the observations by several groups of an association between the expression of proteins containing expanded polyQ tracts and caspase (cysteine aspartate-specific proteases)-dependent apoptotic cell death (32–37).

Though our understanding of the molecular basis of these progressive disorders has improved in recent years, new strategies to modulate the effect of these toxic proteins have been more difficult to develop. Beyond symptomatic treatment, a majority of recent studies investigating potential therapeutic approaches to polyQ disorders have concentrated on suppressing protein aggregation or inhibiting apoptosis. Several studies have shown that co-expression or over-expression of heat shock chaperone proteins that are critical for protein folding can inhibit the toxic effects of the polyQ proteins (38–41) and studies inhibiting caspase-associated cell death have shown that blocking caspase-8 recruitment into polyQ protein aggregates significantly reduces cell death (42). In addition, inhibiting caspase cleavage of the huntingtin protein delays disease onset in transgenic mice (43) and in cell culture (37). An alternative approach to targeting a mutant polyQ protein is to down-regulate its mRNA. Two principal methods have been reported to specifically trigger the degradation of a target transcript: one makes use of antisense oligonucleotides or transcripts and the other uses synthetic ribozyme sequences. Antisense oligonucleotides or transcripts down-regulate gene expression by translational blockade and induction of RNA degradation mainly through the action of the RNase H ribonuclease (11,44). Ribozymes have an enzymatic capability that specifically binds and digests RNA at a defined site (45,46). Although *in vitro* data has been encouraging, the application of these methods has been difficult due to the poor *in vivo* instability of antisense oligonucleotides and the limitation of finding a suitable target sequence for a given ribozyme. Studies aimed at down-regulating transcripts associated with

dominant genetic disorders have included application of antisense technology to Marfan's syndrome (47) and HD (48), and the use of catalytic ribozymes to target rhodopsin transcripts that are associated with autosomal dominant retinitis pigmentosa (49,50). This study now shows the feasibility of targeting an RNA species from an important group of genetic diseases by dsRNA triggered RNAi, including rescue of the caspase-dependent apoptotic cell death induced by polyQ expansions in human cells.

RESULTS

Gene-specific dsRNAs can interfere with expression from *arcag_ngfp* fusion transcripts in *Drosophila* S2 cells

Drosophila embryonic S2 cells have been used extensively as a cell culture system to analyze the RNAi mechanism and its application (51–53). Though several transgenic *Drosophila* models of polyQ diseases have been developed (31,54–56), no cell culture model has been described. To develop a *Drosophila* cell culture model of SBMA, *Drosophila* expression plasmids were constructed to express fusion transcripts containing portions of the human *ar* gene, with CAG tracts of 26, 43 or 106 repeats and the marker green fluorescent protein gene (*gfp*) (Fig. 1A). Northern analysis of mRNA purified from S2 cells transfected with each expression plasmid showed appropriately sized *gfp* containing transcripts (Fig. 3F and data not shown).

Transient transfection of S2 cells with plasmids containing 43 and 106 CAG trinucleotide repeats showed GFP-positive protein aggregates and aggresomes (Fig. 1B). Low-level GFP-associated protein aggregation was observed in S2 cells transfected with pAct.ARCAG43GFP after ~2–3 days; localized aggresomes were seen in 4–5 days. S2 cells transfected with pAct.ARCAG106GFP formed GFP-positive protein aggregates and aggresomes within 24 h. Of those S2 cells transfected, ~4% of pAct.ARCAG43GFP and 35% of pAct.ARCAG106GFP-transfected cells developed GFP-positive protein aggregates 3 days after transfection; this increased to 10% of pAct.ARCAG43GFP-transfected cells and 90% of pAct.ARCAG106GFP-transfected cells 5 days after transfections. These results are consistent with observations in mammalian cells, where trinucleotide length has been shown to correlate with the degree of protein aggregation. Trinucleotide repeats in the range of 40–50 produce proteins that are susceptible to aggregation but this develops over a longer period of time than when a larger repeat expansion is used, represented in this study by the 106 CAG repeat construct (22,23,57). No protein aggregation was seen when the control pAct.GFP or pAct.ARCAG26GFP plasmids were used.

To assess the effect of gene-specific dsRNAs on the expression of the fusion transcripts from each of the pAct.ARCAG_nGFP plasmids dsRNAs of ~80 nt corresponding to portions of the *gfp* gene and the *ar* gene were generated using the appropriate sense and antisense oligomers (Table 1 and Fig. 2); dsRNAs corresponding to the β -galactosidase gene (*LacZ*) and the chloramphenicol acetyl transferase (CAT) gene (*cat*) were used as controls. *Drosophila* S2 cells were co-transfected with each dsRNA molecule and each of the *Drosophila* expression plasmids. Cells were harvested 72 h after initiation of the transfection and assayed for GFP expression (Fig. 3). In all cases the

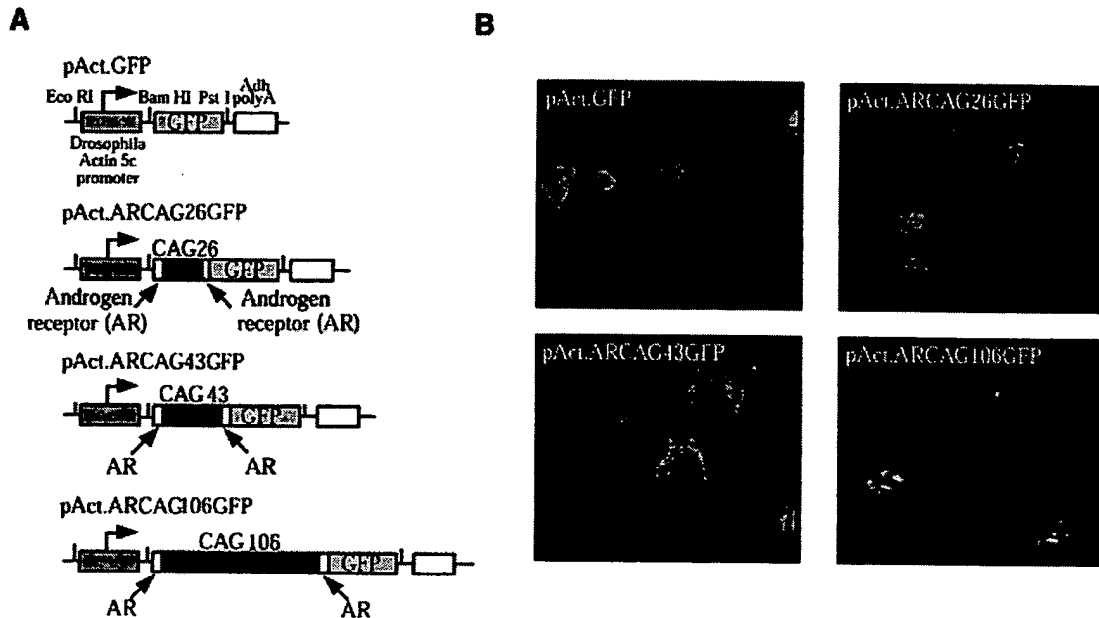


Figure 1. *Drosophila* plasmids expressing *arcag_n:gfp* fusion transcripts and their transient expression in S2 cells. (A) Diagrammatic representations of *Drosophila* expression plasmids used in this study. (B) S2 cells (2×10^6) were transfected with pAct.GFP, pAct.ARCAG26GFP, pAct.ARCAG43GFP and pAct.ARCAG106GFP (2 μ g of plasmid DNA) and were analyzed by deconvolution microscopy 72 h after initiation of transfection. GFP fluorescence is in green and nuclei are counter stained in blue using Hoechst staining.

gfp dsRNA specifically inhibited expression of GFP, including GFP expression from all three of the pAct.ARCAG_nGFP fusion plasmids. The *gfp* dsRNA inhibited GFP expression from pAct.ARCAG26GFP by ~75%, GFP expression from pAct.ARCAG43GFP by ~80% and GFP expression from pAct.ARCAG106GFP by ~85%. The 80% inhibition of GFP expression from the pAct.GFP plasmid is consistent with our previous data (51).

The *ar* dsRNA specifically inhibited GFP expression from the fusion transcripts containing human AR sequences. The *ar* dsRNA induced significant (60–80%) down-regulation of GFP expression from all three of the pAct.ARCAG_nGFP fusion plasmids (Fig. 3A–C). The *ar* dsRNA had no effect on GFP expression from the parental plasmid pAct.GFP, which contains no *ar* sequences (Fig. 3D), and only the dsRNA corresponding to *cat* inhibited CAT expression; the *ar* dsRNA had no significant effect on CAT expression (Fig. 3E).

Northern analysis of mRNA purified from *Drosophila* cells treated with these dsRNAs also demonstrates gene-specific inhibition with a correlation between the number of *gfp* and *ar* containing transcripts and the level of GFP expression observed (Fig. 3F). Using phosphorimage analysis, the levels of the *gfp*-positive and *ar*-positive signals were first normalized using the *Drosophila* glyceraldehyde 3-phosphate dehydrogenase-1 (*gapdh-1*) hybridization signal; the relative levels of the *ar/gfp*-positive transcripts were then compared. The *gfp* dsRNA induced a 70–90% decrease in the *ar/gfp*-positive transcript. The *ar* dsRNA induced a 40% decrease in the level of the *arcag26gfp* transcript; the *arcag43gfp* and *arcag106gfp* mRNA levels were reduced by 60–80% relative to controls.

The effect of dsRNAs containing CAG repeats on transgene expression in *Drosophila* S2 cells

Several studies of RNAi in invertebrate systems have considered the size effects and sequence content of the input dsRNA used to mediate this process (51,58–61), but a transcript containing different numbers of CAG repeats flanked by complex human sequences represents a novel target. To examine the effect of dsRNAs containing highly repetitive sequences, we used dsRNA consisting of 27 CAG repeats (*cag27*) and 27 CAG repeats plus 21 nt from the 5' end of the human AR sequence (*arcag27*) (Table 1 and Fig. 2). Unlike the specific effect seen with the use of complex gene-specific dsRNAs such as the *ar* and *gfp* dsRNAs, the *cag27* dsRNA induced a non-specific inhibition of transgene expression in *Drosophila* cells. Representative data shown in Figure 4 shows the significant inhibition (60–75%) of GFP expression from the pAct.ARCAG_nGFP fusion plasmids (Fig. 4A–C); however, we also consistently saw a decrease in the level of GFP expression from the parental pAct.GFP plasmid of the order of 30–40% (Fig. 4D). We tested a number of preparations of the *cag27* dsRNA, but all had the same effect on GFP expression from pAct.GFP. The addition of 21 nt of the *ar* sequence immediately upstream of the CAG repeat did not improve the specificity of this inhibition, as the *arcag27* dsRNA also reduced GFP expression from the fusion transcripts was inhibited by 70–80% by the *arcag27* dsRNA (Fig. 4A–C). Interestingly, this non-specific interference with transgene expression could, in large part, be alleviated by the use of

In mammalian cells, dsRNAs of over ~80 nt can trigger a variety of cellular responses that result in a non-specific decrease in gene expression and frequently cell death (62). However, recently, we and others have shown that small dsRNAs of ~21–23 nt can mediate a sequence-specific inhibition of gene expression in mouse and human cells (6,7). To determine whether small dsRNAs can specifically interfere with expression of a polyQ protein and thus rescue the increased cell death associated with an expanded CAG tract we co-transfected human HEK-293T cells with mammalian expression plasmids expressing a fusion transcript consisting of a truncated version of the human AR containing either 112 or 19 CAG repeats and *gfp* (pCMV.ARCAG112GFP and pCMV.ARCAG19GFP, respectively) and 22 nt dsRNAs corresponding to either *cat* or *gfp* (Table 1 and Fig. 2). The small dsRNAs used were designed with 5' PO₄ and 2 nt 3'

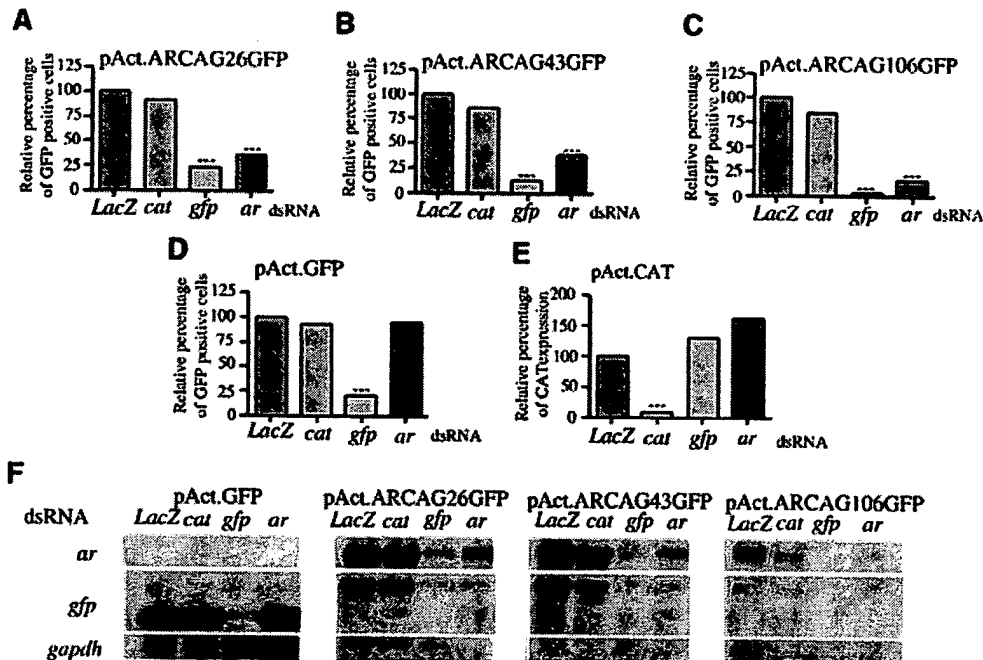


Figure 3. Inhibition of transgene expression by gene-specific dsRNA molecules. dsRNA molecules corresponding to *LacZ*, *cat*, *gfp* or *ar* were co-transfected with either (A) pAct.ARCAG26GFP, (B) pAct.ARCAG43GFP, (C) pAct.ARCAG106GFP, (D) pAct.GFP or (E) pAct.CAT. S2 cells (2×10^6) were transfected with 2 μ g of plasmid DNA and 1 μ g of dsRNA; cells were analyzed 72 h after initiation of transfection. Data shown is representative of at least three independent experiments using each dsRNA and is normalized to the levels of GFP expression observed in those cells co-transfected with the plasmid shown and the control *LacZ* dsRNA, $n = 3$ for each plasmid/dsRNA combination. (F) Northern analysis of poly(A)⁺ RNA ($\sim 2 \mu$ g of each sample) purified from S2 cells transfected with pAct.GFP, pAct.ARCAG26GFP, pAct.ARCAG43GFP and pAct.ARCAG106GFP and either *LacZ*, *cat*, *gfp* or *ar* dsRNA. The northern blot was sequentially hybridized with (i) human *ar*, (ii) *gfp* and (iii) *Drosophila gapdh*-1-specific DNA probes. Statistical analysis was performed by comparison to *LacZ* dsRNA-transfected cells. ** $P < 0.01$, *** $P < 0.001$.

overhangs, a structure that is characteristic of the RNase III (Dicer) enzymatic cleavage that has been associated with RNAi (63). We assayed for GFP expression by deconvolution microscopy (Fig. 5A–F) and assessed toxicity using a fluorescence-activated cell-sort analysis (FACS)-based viability assay of cell death (Fig. 5G and H) 96 h after initiation of transfection. Consistent with previous observations (7), a 22 nt *gfp* dsRNA inhibited GFP expression from both fusion transcripts (Fig. 5E and F); a 22 nt dsRNA corresponding to *cat* had no effect on GFP expression. Further, protein aggregates were clearly evident in pCMV.ARCAG112GFP/*cat* 22 nt dsRNA co-transfected cells whereas there was little or no evidence of protein aggregation in those cells transfected with pCMV.ARCAG112GFP and the 22 nt *gfp* dsRNA. Interference with expression from the ARCAG112GFP transcript mediated by the 22 nt *gfp* dsRNA also significantly reduced the cell death associated with the presence of a polyQ expansion (Fig. 5G) towards control levels (Fig. 5H).

We next tested two 22 nt dsRNAs corresponding to different regions of the human *ar* gene; one region was 5' of the CAG repeat (5'*ar*) and the second target region was 3' of the CAG repeat (3'*ar*). Both dsRNAs significantly reduced the cytotoxic effect mediated by the presence of an AR protein with an expanded polyQ tract (Fig. 5I), with the 22 nt 5'*ar* dsRNA inducing the greatest level of rescue by reducing the

percentage of dead cells to the range seen in control transfections (Fig. 5J). Importantly, both 22 nt *ar* dsRNAs also significantly reduced the levels of caspase-3 activity induced by the ARCAG112 protein. The control *cat* 22 nt dsRNA had no effect on caspase-3 activity indicating the specificity of the effect mediated by the *ar* 22 nt small dsRNAs.

DISCUSSION

dsRNA-mediated silencing of gene expression is increasingly being seen as an important epigenetic mechanism controlling gene expression and as a reverse genetics tool (8,64–66). With the recent description of an RNAi-like mechanism in mammalian somatic cells (6,7) it may now also be possible to eventually exploit dsRNA triggered RNAi as a therapeutic approach akin to current strategies that exploit single-stranded antisense oligonucleotides or transcripts to down-regulate gene expression. In this study, we first assessed the feasibility of such an approach in a model system where RNAi has been relatively well characterized by developing a cell culture model of the dominant genetic disorder SBMA (Kennedy's disease) in *Drosophila* S2 cells. We then used the recently described process of small dsRNA (21–23 nt)-triggered RNAi in mammalian cells to assess whether this could be used to rescue the toxic effects of a truncated human AR protein containing

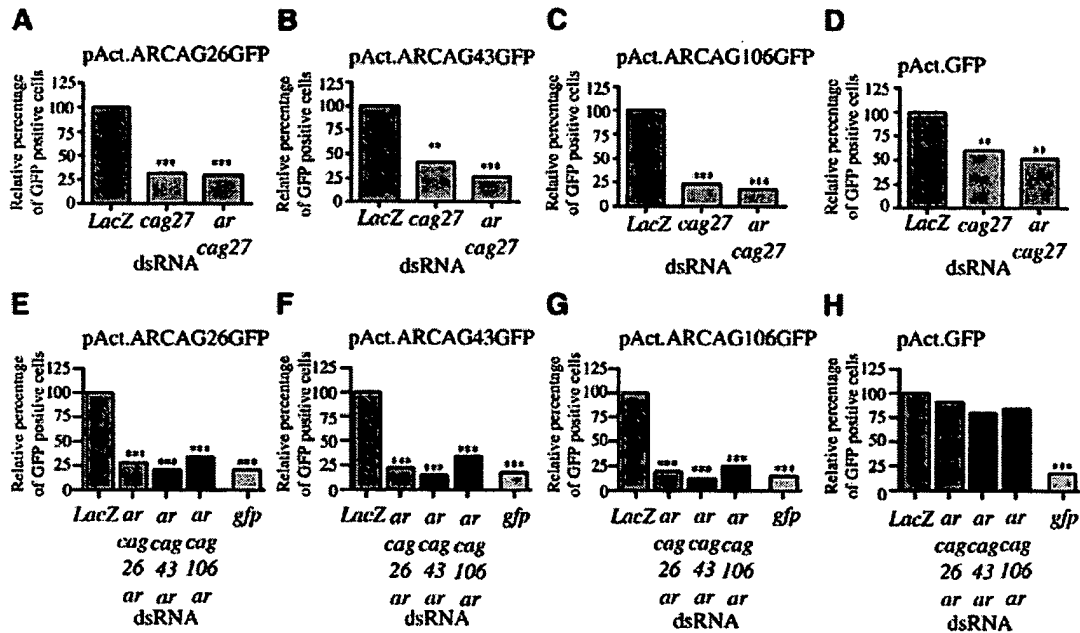


Figure 4. The effect of CAG containing dsRNA molecules on the expression of *arcag_ngfp* fusion transcripts. S2 cells were transfected with pAct.ARCAG26GFP (A and E), pAct.ARCAG43GFP (B and F), pAct.ARCAG106GFP (C and G) or pAct.GFP (D and H) and dsRNA molecules corresponding to: *LacZ* (A–H), 27 CAG trinucleotide repeats (*cag27*) (A–D), 21 nt from the upstream flanking sequence of the human AR plus 27 CAG repeats (*arcag27*) (A–D), *arcag26ar* (E–H), *arcag43ar* (E–H), *arcag106ar* (E–H) or *gfp* (E–H). S2 cells (2×10^6) were transfected with 2 μ g of plasmid DNA and 0.5–1 μ g of dsRNA; cells were analyzed 72 h after initiation of transfection. (A–D) $n = 3$, (E–H) $n = 5$, data shown is normalized to the levels of GFP expression observed in those cells co-transfected with the plasmid shown and the control *LacZ* dsRNA. Statistical analysis was performed by comparison to *LacZ* dsRNA-transfected cells. ** $P < 0.01$, *** $P < 0.001$.

an expanded polyQ tract. In summary, in *Drosophila* cells we saw dsRNA-mediated gene-specific, but not allele-specific, inhibition of transcripts encoding a truncated version of the human AR with different trinucleotide repeat lengths. In human cells, small dsRNAs specifically inhibited similar truncated *ar*-containing transcripts and, further, this inhibition protected cells from the cytotoxic effects associated with the accumulation of truncated AR protein containing an expanded polyQ tract.

In invertebrates such as *C.elegans* and *Drosophila* dsRNA-mediated RNAi is most effectively triggered by RNA duplexes of over ~80 nt. However, several recent studies have shown that the larger input dsRNA is cleaved into fragments of ~21–25 nt that act as guides for the enzymatic complex required for the degradation of the target mRNA (53,58,59,61,63,67,68). It is this observation which may explain our detection of gene-specific, but not allele-specific, inhibition as cleavage of the *arcag26ar*, *arcag43ar* or *arcag106ar* dsRNAs to dsRNAs of ~21–25 nt will likely result in a loss of any duplex that distinguishes these from one another. This obviously has significance for the application of this methodology to dominant genetic disorders where the only distinguishing feature is a trinucleotide expansion. In the case of X-linked SBMA, which is mono-allelic, this is potentially less of an issue though androgen insensitivity may be exacerbated, but where loss of function of the normal allele may have a broader clinical impact, this observation will need to be considered. Though the *arcag_nar* dsRNAs did not confer allele

specificity, the addition of larger *ar* flanking sequences did protect against the non-specific effects seen when dsRNAs generated from oligoribonucleotides with 27 CAG repeats were used. There are several possible reasons for this, one is that the CAG repeat results in a complex secondary folding that generates a toxic duplex; however, we saw no overt cytotoxicity and no effect on *gapdh-1* mRNA levels (data not shown). Alternatively, there is the potential that the small dsRNAs generated from the *cag27* and *arcag27* dsRNAs can target a broader range of transcripts that contain a small repeat, resulting in decreased expression from the parental plasmid (pAct.GFP) as well as the transcripts we were aiming to target. However, in both cases the *cag27* and the *arcag27* dsRNAs did induce a greater inhibition of the *arcag_nar* transcripts than the parental plasmid suggesting at least a level of targeted degradation was occurring.

Sequence-specific dsRNA mediated interference of gene expression has only recently been observed in mammalian cell culture systems (6,7). We have now shown that the same mechanism has the potential to specifically target a transcript associated with a dominant genetic disorder and to rescue the adverse cellular changes associated with the expression of a transcript containing a trinucleotide expansion. Though the inhibition mediated in this study used a transient co-transfection system, there is evidence that the same small dsRNA methodology can be used to inhibit endogenous gene expression (6; and N.J.Caplen, unpublished data). As yet, we know little about the optimal characteristics of the small dsRNAs that mediate this

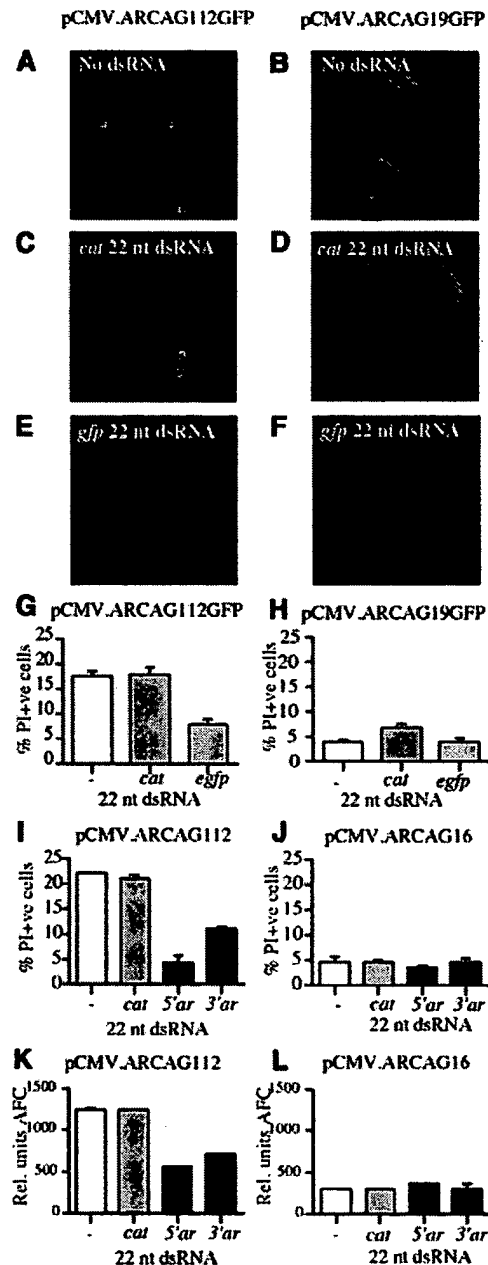


Figure 5. Specific inhibition of *arcag,ar* transcripts by small dsRNAs in human HEK-293T cells and rescue of polyQ-induced cytotoxicity. (A–H) HEK-293T cells (1×10^6) were transfected with pCMV.ARCAG112GFP (A, C, E and G) or pCMV.ARCAG19GFP (B, D, F and H) and dsRNAs corresponding to *cat* (C, D, G and H) or *gfp* (E–H) and were assayed for GFP expression and general cell morphology by deconvolution microscopy (A–F) (GFP fluorescence is in green and nuclei are counter stained in blue using Hoeschst staining) or cell death by PI staining (G and H; data are shown as means \pm SD, $n = 3$) 72 h after initiation of transfection. (I and J) HEK-293T cells (1×10^6) were transfected with pCMV.ARCAG112 (I and K) or pCMV.ARCAG16 (J and L) and dsRNAs corresponding to *cat* and two different regions of the human AR (*5'ar* and *3'ar*); cells were assayed 72 h after initiation of transfection for cell death by PI staining (I and J) and caspase-3 activity (relative fluorescence units of AFC released/min/ μ g protein) (K and L). Data shown are means \pm SD from three independent transfections.

response in mammalian cells or the optimal nature of the target mRNA. All three of the experimental 22 nt dsRNAs used in this study inhibited expression of the target transcript, though we saw some variation in the degree of rescue mediated by the two *ar* dsRNAs, with the *5'ar* dsRNA proving more effective than the *3'ar* dsRNA. Both of these dsRNAs have similar sequence complexity and structure. However, the *5'ar* dsRNA targeted a region overlapping the start of the *ar* open-reading frame ~ 160 nt from the start of the CAG tract whereas the *3'ar* dsRNA targeted a sequence ~ 90 nt downstream of the CAG tract and it may be these features of the target sequence that explain the slight variations we observed. We are currently conducting studies to determine more precisely the parameters that affect interference by small dsRNAs in mammalian cells and importantly we now need to determine if this mechanism is also present in a wider range of cell types than have been tested so far, including neuronal cells. This study demonstrates for the first time the feasibility of targeting a disease-associated transcript by dsRNA-mediated RNAi. As our understanding of the mechanism of RNAi improves it is now increasingly likely that this methodology could be developed not only as an effective reverse genetics tool in a wide range of species but also as a gene therapy approach.

MATERIALS AND METHODS

Plasmids and generation of dsRNA

The construction of pAct.GFP and pAct.CAT have been previously described by Caplen *et al.* (51). The plasmids pAct.ARCAG26GFP, pAct.ARCAG43GFP and pAct.ARCAG106GFP were constructed by insertion of a previously constructed cassette containing 178 bp (nucleotides 524–702) and 207 bp (nucleotides 1345–1552) of the human AR 5' and 3' of 112 CAG repeats in frame with *egfp* (Clontech) downstream of the *Drosophila* actin 5c promoter in pAct.Adh (L.Chen, Indiana University, Bloomington, IN). Clones were screened for contraction or expansions of the CAG repeat during cloning and three clones with 26, 43 and 106 CAG trinucleotide repeats were identified (Fig. 1). The pAR_(N) constructs, pCMV-ARCAG16 and pCMV-ARCAG112 have been described previously by Merry *et al.* (23). The pCMV-ARCAG19GFP and pCMV-ARCAG112.GFP plasmids were generated by sub-cloning *Xba*I–*Eco*R1 fragments from pAR_(N) into pEGFP-N1 (Clontech) generating an in-frame fusion cDNA consisting of a truncated form of the human AR and enhanced GFP (EGFP).

Single-stranded, gene-specific sense and antisense RNA oligomers corresponding to the β -galactosidase (*LacZ*) gene, the CAT (*cat*) gene, the GFP protein (*gfp*) gene and the human *ar* gene (sequences shown in Table 1) were synthesized using 2'-O-(triisopropyl)silyloxymethyl chemistry by Xeragon AG. Sense and antisense oligoribonucleotides consisting of 27 CAG trinucleotides and 27 CAG trinucleotide repeats plus 21 nt from the 5' region of the human AR immediately adjacent to the CAG tract (see Table 1 for sequences) were also chemically synthesized. To form dsRNA molecules 100 μ g of each complementary single-stranded RNA (ssRNA) oligomer in 10 mM Tris–Cl pH 7.0, 20 mM NaCl (total volume 300 μ l) was heat denatured at 95°C in a water bath and then allowed to anneal slowly (in the water bath) for ~ 18 h. The dsRNAs

were ethanol precipitated and re-suspended in RNase free water at a concentration of ~0.5 mg/ml. Double-stranded RNA molecules corresponding to *arcag26ar*, *arcag43ar* and *arcag106ar* were generated by sub-cloning each cassette as an *EcoRI*–*Bss*HII fragment into pCMV-BK (Stratagene). Sense and antisense RNAs were synthesized for each cassette using standard T3 and T7 RNA polymerase *in vitro* transcription reactions (Megascript; Ambion) after preparation of a *PvuII*–*NheI* fragment which included non-homologous plasmid sequences. Double-stranded RNAs were generated as above; after precipitation flanking ssRNAs derived from non-homologous plasmid sequences were removed using Ribonuclease T1 in 0.3 M NaCl. The integrity and change in mobility of all of the dsRNAs were confirmed by gel electrophoresis.

Cell culture and nucleic acid transfections

S2 cells were grown in DESTTM Medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS). Cells were passaged every 2–3 days to maintain exponential growth. S2 cells were transfected using either the cationic lipid CellFectin (Life Technologies) using an adaptation of the manufacturer's protocol. Briefly, cells were seeded and allowed to settle overnight, nucleic acid (plasmid and/or dsRNA) was complexed with lipid at a weight to weight ratio of 1:6 in DES medium without supplementation. The complex was incubated at room temperature for 15 min and then added to cells from which normal growth medium had been removed. After overnight incubation an equal volume of DES medium plus 20% FBS was added to the cell/lipoplex mixture. HEK-293T cells were maintained in DMEM medium (Gibco BRL) supplemented with 10% FBS. Cells were plated at a density of 60% in six-well culture dishes the day prior to transfection. Transfection with 1 µg of DNA was carried out using Lipofectamine Plus following the manufacturer's protocol (Gibco BRL) plus 0.5 µg of dsRNA.

Transgene expression

GFP expression was assessed by FACS analysis or deconvolution microscopy. For FACS analysis (FacsCaliber; Becton Dickinson) pAct.CAT-transfected cells were used to gate for forward scatter and side scatter. The percentage of GFP-positive cells was determined by gating against pAct.CAT-transfected cells with M1 falling between 10 or 20 and 9910 (FL-1), the geometric mean was used as a measure of the relative intensity of fluorescence. 10 000 non-gated events were acquired for each sample. For deconvolution microscopy S2 and 293 cells were either spun onto slides or grown on coverslips or two-well chamber-slides (Nunc). At the indicated times the cells were fixed in 4% paraformaldehyde for 15 min at room temperature then washed three times in PBS. All cells were stained with 1 µg/ml Hoechst 33342 (Sigma) in PBS for 10 min. Deconvolved images were produced on an Olympus microscope using a 60× water-immersion objective and Deltavision software (Applied Precision) on a Silicon Graphics workstation. Multiple optical sections 0.2 µm thick were analyzed.

To directly measure the toxicity of expanded polyglutamine in the context of truncated AR we employed a FACS-based survival assay. HEK-293T cells were harvested with trypsin,

gently pelleted by centrifugation and resuspended in PBS with 0.5% serum ice at a concentration 10⁶/ml. The cells were stained with 1 µg/ml propidium iodide (PI) (Sigma), gently vortexed and incubated for 15 min at room temperature in the dark. 50 000 non-gated events were acquired for each sample (Beckman Coulter XL instrument and software package used for analysis). Results are expressed as a percentage of PI-negative (viable) cells (FL-2 channel) relative to total GFP-positive (transfected) cells (FL-1 channel).

CAT expression was assessed using an ELISA-based method (Roche Biochemicals). Total protein was determined using the Bradford method and the micro-assay protocol adapted for use in 96-well micro-titer plates (Bio-Rad). Absorption readings (*A*₅₉₅) were converted to absolute amounts using a bovine serum albumin (Sigma) (0.625–10 µg) standard curve after subtraction of background values. Statistical analyses were conducted using an unpaired *t*-test (SatView) for comparison of groups. The null hypothesis was rejected at *P* > 0.05.

RNA analysis

Total RNA was isolated using GTC extraction and poly(A)⁺ RNA selected using Oligo dT cellulose (Ambion). The poly(A)⁺ RNA was subjected to DNase digestion to remove any trace contamination of plasmid DNA and analyzed by electrophoresis (1.2% agarose, 1× MOPS, 5.0% formaldehyde), northern blot transfer and hybridization (Ambion) at 42°C with ³²P-labeled random-primed DNA probes. A human AR gene-specific probe (148 bp) was prepared by PCR amplification using pCMV.AR112GFP as template and the following oligonucleotides (*ar* forward, 5'-gga agt gca gtt agg gct g-3'; *ar* reverse, 5'-cag cag cag caa act ggc g-3'). The *gfp*, and *Drosophila gapdh-1* DNA probes have been previously described by Caplen *et al.* (51). In all cases, filters were washed at high stringency (0.2× SSC) and subjected to autoradiography. The intensities of the hybridization signals were obtained using a Fuji BAS1500 phosphorimager (Fuji Photo Film Co.) and pixel densities calculated using Image reader 1.4 and ImageGauge 3.0 (Fuji Photo Film Co.).

Caspase-3 activity

To quantitate caspase-3 activity, 10⁶ cells were washed with cold PBS and harvested by scraping at the indicated times post-transfection. The cells were pelleted at 400 g for 5 min then re-suspended in lysis buffer (10 mM Tris, pH 7.3, 10 mM NaH₂PO₄, 150 mM NaCl, 1% Triton X-100). Protein concentration was determined using the DC protein assay reagent (Bio Rad). 100 mg of cell extract was incubated with 50 mM of fluorometric substrate Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (DEVD-AFC) (ApoTarget kit; Biosource International) in a total volume of 100 µl of reaction buffer (10 mM HEPES, pH 7.4, 50 mM NaCl, 0.5 mM EDTA, 0.1% CHAPS, 10% glycerol, 10 mM DTT) in the dark for 1 h at 37°C. DEVDase activity was determined by measuring the liberation of AFC using a Cytofluor II multiwell plate reader (PerSeptive Biosystems) with excitation and emission wavelengths of 420 and 520 nm, respectively. Caspase-3 specificity was established by blocking activity with the inhibitor DEVD-CHO at 10 mM.

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